# THE ISOLATION AND ENUMERATION OF ACTINOMYCETES FROM WATER SAMPLES

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This paper reports the results obtained during investigations under project number 66.9.GD, and forms part of a continuing study into taste and odour problems being carried out by the Ontario Water Resources Commission, Division of Research.

# INTRODUCTION

There is considerable interest in the occurrence of taste and odour problems in water supplies. The OWRC became concerned with these problems and their relation to the presence of members of the Actinomycete group. Indeed, these may be the only organisms responsible for the production of such tastes and odours; Silvey et al (1) have cited instances where cultures of algae producing the odours under laboratory conditions, were found to contain Actinomycetes.

Only three Actinomycete genera are likely to be of importance in the examination of water supplies, namely Streptomyces, Nocardia and Micromonospora. A wide variety of methods for the isolation and enumeration of these forms have been reported, but not all are applicable to routine studies. This investigation was undertaken to develop a technique which could be used in the routine analysis of water samples.

## GENERAL

Most species of Actinomycetes are relatively slow-growing in comparison to bacteria and fungi. Their isolation involves the suppression of these other microorganisms which may be present in the same environment, combined with an enhancement of their own growth. One of the simplest methods (2) is to plate out a water sample (1 to 5 ml) on to nutrient agar containing twice the normal amount of agar; the competing organisms become dehydrated, whereas the Actinomycetes can survive. But this can only be used in circumstances where they are in relatively large numbers (in excess of 10 to 30 per ml), in order to obtain an accurate count. Actinomycete isolation may also be accomplished by providing them with nutrient sources which other microflora have difficulty in metabolizing, and/or suppressing these competing forms by the addition of one or more antibiotics. There appears to be general agreement that Actidione is the antibiotic of choice for the suppression of fungal forms.

In this study, six media were evaluated for their ability to support the growth of members of the three important genera in pure culture. The optimum medium, on the basis of this evaluation, was then used to test the various methods of

isolation which have been recommended. The technique which gave the best results could then be adopted for use in the examination of field samples.

## Part A DETERMINATION OF THE OPTIMUM ISOLATION MEDIUM

### Experimental

(a) Cultures The cultures of Actinomycetes used, were isolated in pure culture on yeast extract/glucose agar (3), from nutrient agar plates which had been inoculated with bottom mud samples of Lake Ontario collected in the course of another study. Differentiation between the genera was made according to the scheme adapted from Waksman and Henrici (4), based on fragmentation of the mycelium and the arrangement of the conidia, when slide preparations were viewed microscopically. Species identification was not carried out, but four different species from each genus was selected for the evaluation, on the basis of gross morphology produced on the same medium under identical conditions. Thus, twelve different species were tested in all: Streptomyces, strains 4, 15, 16, 17, Nocardia, strains 1, 2, 6, 10 and Micromonospora, strains 19, 20, 23 and 28.

(b) Preparation of Inocula For the tests, a loopful of stock culture was transferred to 5 ml tryptone/yeast extract broth (5) and grown stationary at room temperature (22°C) for 14 days. The medium was then decanted, the growth was washed and then washed with sterile distilled water into a 125 ml conical flask containing 100 ml sterile, distilled water. The flask was stoppered and shaken vigorously 25 times and the contents allowed to settle, whereupon the shaking was repeated. After settling, the supernatant was used as an inoculum, being added dropwise (0.05 ml) on to the surface of each test medium. In this way six different Actinomycetes could be tested simultaneously on a single plate of test medium.

(c) Test Media (Appendix 1) The following five media were tested for their ability to support the growth of the twelve test strains: sodium caseinate agar (6) egg albumin agar (7), casein glycerol agar (8), glycerol/arginine agar (9) and AGS medium (10). These media were made up both with and without Actidione*, even if the medium as originally formulated did not contain the antibiotic and the growth tests were run in parallel. This would indicate whether the addition had any deleterious effect on the Actinomycetes themselves. The Actidione was prepared as a stock solution containing 1 mg/ml,

and autoclaved at 121°C for 15 minutes; it was added to the medium prior to plating to give a concentration of 0.05 mg/ml of medium. All plates were dried for 15 minutes at 35°C before use.

## RESULTS

The results of a series of experiments are shown in Tables 1 a, b, and c. The optimum medium for all strains was found to be sodium caseinate agar, followed by egg albumin agar; although AGS medium gave almost equal growth of Streptomyces and Nocardia strains, it did not produce an equivalent yield of the Micromonospora species. The addition of Actidione appeared to have very little effect on the growth. It can be seen that a final reading was made at 7 days; after this point, satellite colonies began to appear around the original colonies, especially in the case of Streptomyces species, and there was very little change in the size or number of the original colonies.

* Actidione - Upjohn & Company, Kalamazoo, Michigan.

**TABLE 1a**

Growth of Streptomyces species in different media,
after incubation for 1 week at room temperature

| Medium / Strain | Test | Sod. Caseinate +ACT* | Sod. Caseinate -ACT** | Kuster & Williams +ACT | Kuster & Williams -ACT | Glycerol/Arginine +ACT | Glycerol/Arginine -ACT | A.G.S. +ACT | A.G.S. -ACT | Egg Albumin +ACT | Egg Albumin -ACT |
|---|---|---|---|---|---|---|---|---|---|---|---|
| S4 | A | 4*** | 4 | 4 | 4 | 2 | 2 | 4 | 4 | 4 | 4 |
| | B | 4 | 4 | 3 | 3 | 2 | 2 | 4 | 4 | 4 | 3 |
| S15 | A | 4 | 4 | 4 | 4 | 3 | 3 | 4 | 4 | 3 | 3 |
| | B | 4 | 4 | 4 | 4 | 2 | 2 | 4 | 4 | 3 | 3 |
| S16 | A | 4 | 4 | 3 | 3 | 2 | 2 | 3 | 3 | 4 | 4 |
| | B | 3 | 4 | 3 | 4 | 2 | 2 | 4 | 4 | 3 | 3 |
| S17 | A | 4 | 4 | 3 | 3 | 2 | 2 | 2 | 2 | 4 | 4 |
| | B | 4 | 4 | 3 | 3 | 1 | 1 | 3 | 3 | 3 | 3 |

* - medium with Actidione
** - medium without Actidione
*** - Growth is graded from 1 = poor to 4 = excellent

TABLE 1b

Growth of Nocardia species in different media
after incubation for 1 week at room temperature

| Medium | | Sod. Caseinate | | Kuster & Williams | | Glycerol/Arginine | | A.G.S. | | Egg Albumin | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | Test | +ACT* | -ACT** | +ACT | -ACT | +ACT | -ACT | +ACT | -ACT | +ACT | -ACT |
| N1 | A | 4*** | 4 | 4 | 4 | 2 | 2 | 4 | 4 | 4 | 4 |
| | B | 4 | 4 | 3 | 3 | 2 | 2 | 4 | 4 | 3 | 4 |
| N2 | A | 4 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 3 | 3 |
| | B | 3 | 4 | 3 | 3 | 2 | 2 | 2 | 2 | 3 | 3 |
| N6 | A | 4 | 4 | 3 | 3 | 2 | 2 | 3 | 3 | 3 | 3 |
| | B | 4 | 4 | 3 | 3 | 1 | 1 | 4 | 4 | 3 | 3 |
| N10 | A | 4 | 4 | 3 | 3 | 2 | 2 | 4 | 4 | 3 | 3 |
| | B | 4 | 4 | 3 | 3 | 2 | 2 | 4 | 4 | 3 | 3 |

* - medium with Actidione

** - medium without Actidione

*** - Growth is graded from 1 = poor to 4 = excellent

TABLE 1c

Growth of Micromonospora species in different media,
after incubation for 1 week at room temperature

| Medium / Strain | Test | Sod. Caseinate +ACT* | Sod. Caseinate -ACT** | Kuster & Williams +ACT | Kuster & Williams -ACT | Glycerol/Arginine +ACT | Glycerol/Arginine -ACT | A.G.S. +ACT | A.G.S. -ACT | Egg Albumin +ACT | Egg Albumin -ACT |
|---|---|---|---|---|---|---|---|---|---|---|---|
| M19 | A | 4*** | 4 | 1 | 1 | 2 | 2 | 2 | 2 | 3 | 3 |
| | B | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| M20 | A | 4 | 4 | 2 | 2 | 1 | 1 | 2 | 2 | 3 | 3 |
| | B | 3 | 4 | 2 | 2 | 1 | 1 | 1 | 1 | 3 | 3 |
| M23 | A | 4 | 4 | 2 | 2 | 1 | 1 | 2 | 2 | 3 | 3 |
| | B | 4 | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 |
| M28 | A | 4 | 4 | 1 | 1 | 1 | 1 | 2 | 2 | 4 | 4 |
| | B | 4 | 4 | 2 | 2 | 0 | 0 | 2 | 2 | 3 | 3 |

* - medium with Actidione

** - medium with Actidione

*** - growth is graded 1 = poor to 4 = excellent

## Part B DETERMINATION OF THE OPTIMUM ISOLATION METHOD

### Experimental

(a) Cultures One species of each genus was selected for this part of the study. These were S16, M20 and N1 from Part A. They were maintained as previously described.

(b) Preparation of Inocula A stock culture was inoculated on to a yeast extract/glucose agar slant, and incubated at room temperature for 14 days. To prepare the spore suspensions, loopfuls of the sporulating mycelium were removed and placed in 200 ml sterile distilled water in a 500 ml flask, which was then shaken vigorously 25 times; allowed to settle and again shaken vigorously. In the case of the Nocardia and Micromonospora species, some of the vegetative mycelium was' also removed. After settling, the supernatant was decanted into a fresh, sterile flask. The number of spores (and possible also, mycelial fragments) per ml of the stock suspensions was then determined by a standard plate count technique. Aliquots of each suspension could then be diluted to contain approximately the numbers required in each test. Suspensions were stored at +4$^{o}$C for the duration of the experiments.

(c) Media The medium used for all tests was that found to be the optimum in Part A i.e. sodium caseinate agar plus Actidione, unless otherwise stated.

(d) Test Isolation Methods Although Morris (11) has found up to 300 organisms per ml in waters where a taste and odour problem exists, the numbers of Actinomycetes in surface waters would generally be such that some method of concentration would be required. The most suitable method is membrane filtration, (0.45 $\mu$ Millipore filter) and there are several ways in which this can be applied.

After filtration of the water sample, the membrane may be:

a) placed directly on the surface of the medium and incubated at room temperature.

b) placed face-downwards in the surface of the medium and incubated in this position for 2 hours at 35°C. The membrane is then carefully removed and the plate incubated at room temperature (12).

c) transferred to a stoppered conical flask (50 ml) along with a number of sterile glass boiling beads, and 5 ml dilution water (13). After shaking vigorously 100 times, the organisms are present in the fluid, from which 0.1 ml samples may be plated on the medium. (14)

d) directly cultured on a medium which has been recommended by Masschelein specifically for Actinomycetes isolation using membrane filters (15).

Thus, for each species, four identical 100 ml suspensions were made in dilution water, by diluting the standard spore suspension to the required level (50-100 Actinomycetes per 100 ml approximately); each was filtered through a membrane filter, which was then treated in one of the four above ways. A control count of the standard suspension was made by the plate count technique at the time of each test, allowing a comparison to be made between numbers recovered on the membranes and the actual number in the samples. Three separate tests were run for each species. The counting was carried out with the assistance of a stereoscopic microscope, using oblique lighting.

## RESULTS

Tables 2, 3 and 4 show the recoveries obtained by the various methods. The control counts of the standard suspensions were found not to vary significantly from test to test. For each species therefore, the average of the control counts was used to calculate the actual number of Actinomycetes present in the test suspension. The fact that

there was no variation in count, indicates that the number of Actinomycetes suspended in tap water does not change significantly over a period of one week at +4°C. Masschelein's method does enable the recovery of Nocardia and Streptomyces strains but growth of the Micromonospora strain was very poor. It apparently does not give an accurate estimation of numbers, and the plates are also very readily contaminated with aerial molds when opened for counting purposes. Removal of the filter after incubation face downwards, is not an efficient isolation method, as supported in studies by the WRA (16). At these relatively low levels of Actinomycetes, the removal of the organisms from the filter, subsequent to filtration, does not enable accurate enumeration, since there is a dilution factor of 1/5 involved, and only 0.1 ml of the resultant solution can be successfully plated; the numbers then obtained are below the accuracy limit of 30 colonies per plate. With greater numbers present (500-1,000 per 100 ml) recoveries are erratic, sometimes being greater than theoretical, possibly due to a breaking up of clumps of spores during the manipulations. The extra steps involved and the fact that the economical, 47 mm Millipore culture plates cannot be used, are added disadvantages to this method. The method whereby the filter is cultured directly, gave the best results in this series of experiments.

TABLE 2

Numbers of Streptomyces (Strain 4) recovered by different methods from 100 ml of a suspension containing 69 organisms per 100 ml by standard plate count.

| Method of Recovery | Test 1 | | Test 2 | | Test 3 | |
|---|---|---|---|---|---|---|
| | 7 days | 14 days | 7 days | 14 days | 7 days | 14 days |
| a) Direct culture of filter | 44 | 44 | 72 | 71 | 57 | 58 |
| b) Face down incubation and removal of filter | 21 | 21 | 21 | 22 | 7 | 8 |
| c) Removal of cells from filter with glass beads | 5 | 5 | 20 | 15 | 20 | 20 |
| d) Masschelein's medium | 12 | OWM | 6 | OWM | 0 | OWM |

OWM = overgrown with molds

TABLE 3

Numbers of Nocardia (Strain 6) recovered by different methods from 100 ml of a suspension containing 140 organisms per 100 ml by standard plate count.

| Method of Recovery | Test 1 | | Test 2 | | Test 3 | |
|---|---|---|---|---|---|---|
| | 7 days | 14 days | 7 days | 14 days | 7 days | 14 days |
| a) Direct culture of filter | 121 | 123 | 123 | 123 | 111 | 111 |
| b) Face down incubation and removal of filter | 67 | 70 | 91 | 92 | 67 | 67 |
| c) Removal of cells from filter with glass beads | 60 | 60 | 70 | 70 | 90 | 100 |
| d) Masschelein's medium | 10+ | OWM* | 7 | 7 | 13 | OWM |

OWM = overgrown with molds

TABLE 4

Numbers of Micromonospora (Strain 23) recovered by different methods from 100 ml of a suspension containing 84 organisms per 100 ml by standard plate count.

| Method of Recovery | Test 1 | | Test 2 | | Test 3 | |
|---|---|---|---|---|---|---|
| | 7 days | 14 days | 7 days | 14 days | 7 days | 14 days |
| a) Direct culture of filter | 52 | 64 | 43 | 60 | 61 | 71 |
| b) Face down incubation and removal of filter | 20 | 23 | 8 | 19 | 32 | 32 |
| c) Removal of cells from filter with glass beads | 3 | 3 | 0 | 0 | 28 | 37 |
| d) Masscheleins medium | 0 | 0 | 0 | 0 | 0 | 0 |

In all methods, it is necessary that the surface of the plates be dried, and at least 15 minutes drying at 35°C should be allowed. Although some colonies were generally visible at 3 days, there was an increase in numbers up to seven days, due to the development of the slower growing cells. However, after seven days there was no further appreciable increase except in the case of the Micromonospora strain; this is in accord with the findings of the R.A. Taft SEC (6) where counts were made after 6-7days at 25°C. Upon longer incubation of Streptomyces and Nocardia species, satellite colonies tend to appear around original colonies and this tends to lead to confusion in counts made after seven days.

The Streptomyces and Nocardia colonies are quite simple to identify when they appear in mixed cultures with bacteria, due to aerial mycelium production, dull wrinkled surface and/or the discoloration produced in the medium; but Micromonospora colonies are not quite so distinct, and it is sometimes necessary to make smears of specimen colonies to ascertain identity on the basis of microscopic morphology. This would particularly be the case for mixed cultures, as would occur in the examination of water samples in the field. In such cases, the small Micromonospora colonies could easily be completely obscured by other colonies.

## CONCLUSIONS

Sodium caseinate agar plus Actidione was the optimum medium of those tested, for the isolation from pure suspensions of the three genera of Actinomycete likely to be of importance in water supplies. Where the isolation of relatively low numbers are required, a suitable quantity of the water (usually 100 ml) may be filtered through a 0.45 μ Millipore filter, which is then cultured on the surface of the medium. Where larger numbers occur, the same technique may be applied to a suitably diluted sample of water.

## RECOMMENDATIONS

On the basis of this study, the following recommendations are submitted:

1. The membrane filter method described above for the examinations of water samples for the presence of Actinomycetes should be tested in the field, especially where a taste and odour problem occurs in the water. Since the method enables isolation of low numbers, it is able to furnish information on the normal levels of these organisms in various waters.

2. There is a need for an improved medium for the isolation of Micromonospora species, to obviate the overgrowth of these small, very slow-growing colonies by competing forms.

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## APPENDIX

### 1. SODIUM CASEINATE AGAR

| | |
|---|---|
| Glucose | 1.0 gm |
| Sodium caseinate | 2.0 gm |
| $K_2HPO_4$ | 0.2 gm |
| $MgSO_4 7H_2O$ | 0.2 gm |
| $FeSO_4 7H_2O$ | 0.01 gm |
| Agar | 15.0 gm |
| Distilled Water | 1,000 ml |

pH 7.3 after sterilization

### 2. EGG ALBUMIN AGAR

| | |
|---|---|
| Glucose | 1.0 gm |
| Soluble egg albumin | 0.25 gm |
| $K_2HPO_4$ | 0.5 gm |
| $MgSO_4 7H_2O$ | 0.2 gm |
| $Fe_2(SO_4)_3 7H_2O$ | 0.01 gm |
| Agar | 15.0 gm |
| Distilled water | 1,000 ml |

pH 7.1 after sterilization

### 3. CASEIN/GLYCEROL AGAR

| | |
|---|---|
| Casein | 0.3 gm |
| Glycerol | 10.0 gm |
| $KNO_3$ | 2.0 gm |
| $K_2HPO_4$ | 2.0 gm |
| $MgSO_4$ | 0.05gm |
| $FeSO_4$ | 0.1 gm |
| $CaCO_3$ | 0.02gm |
| NaCl | 2.0 gm |
| Agar | 18 gm |
| Distilled water | 1,000 ml |

pH 7.0-7.2 after sterilization

4. GLYCEROL/ARGININE AGAR

| | | |
|---|---|---|
| Glycerol | 20 | gm |
| L-arginine | 2.5 | gm |
| NaCl | 1.0 | gm |
| $CaCO_3$ | 0.1 | gm |
| $FeSO_4 7H_2O$ | 0.1 | gm |
| $MgSO_4 7H_2O$ | 0.1 | gm |
| Agar | 20 | gm |
| Distilled water | 1,000 | ml |

5. A.G.S. MEDIUM

| | | |
|---|---|---|
| Arginine monohydrochloride | 1.0 | gm |
| Glycerol | 12.5 | gm |
| $K_2HPO_4$ | 1.0 | gm |
| NaCl | 1.0 | gm |
| $MgSO_4 7H_2O$ | 0.5 | gm |
| $Fe_2(SO_4)_3 6H_2O$ | 0.01 | gm |
| $CuSO_4 5H_2O$ | 0.001 | gm |
| $ZnSO_4 7H_2O$ | 0.001 | gm |
| $MnSO_4 H_2O$ | 0.001 | gm |
| Agar | 15 | gm |
| Distilled | 1,000 | ml |

pH 6.9-7.1

All culture media were autoclaved at 121°C for 20 minutes.

6. ACTIDIONE STOCK

| | | |
|---|---|---|
| Actidione | 100 | mg |
| Distilled water | 100 | ml |

Autoclave at 121°C for 15 minutes

7. MASSCHELEIN'S MEDIUM

| | | |
|---|---|---|
| Bacto Sabouraud Maltose agar | 6.5 | gm |
| Bromocresol green | 0.1 | gm |
| Distilled water | 100 | ml |

Autoclave at 121°C for 15 minutes